Overlapping Distribution of Autoantibody Specificities in Paraneoplastic Pemphigus and Pemphigus Vulgaris

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Paraneoplastic pemphigus is an autoimmune bullous skin disease in which autoantibodies immunoprecipitate a characteristic antigenic complex. The objective of this study was to analyze by immunoblotting and immunoelectron microscopy the autoimmune response in five patients with clinical and immunohistologic features typical of paraneoplastic pemphigus. In a first series of experiments, immunoblotting and immunoelectron microscopy were performed using anti-human whole Ig. Although immunoblotting results were consistent with the autoantibody specificities previously described in paraneoplastic pemphigus sera, immunoelectron microscopy demonstrated the presence of Ig deposits on desmosomal plaques, on hemidesmosomes and, surprisingly, on both the extracellular part of desmosomes and the keratinocyte plasma membrane.

In a second series of experiments, immunoblotting and immunoelectron microscopy were carried out using anti-human IgG subclasses. The major observation was that two sera contained, in addition to the anti-desmoplakins I–II, anti–185-kD and anti–230-kD autoantibodies, autoantibodies that stained the desmoglea by indirect immunoelectron microscopy and bound to a 130-kD polypeptide by immunoblotting. One serum was particularly demonstrative: IgG1 bound to the 250- and 220-kD bands corresponding to desmoplakins I and II on immunoblots and to the desmosomal plaques of keratinocytes in immunoelectron microscopic preparations; IgG3 recognized a 185-kD immunoblotting band and hemidesmosomes and desmosomal plaques by immunoelectron microscopy; IgG4 bound to the 130-kD immunoblotting band of pemphigus vulgaris and labeled the desmoglea and the keratinocyte plasma membrane by immunoelectron microscopy.

These results demonstrate that the paraneoplastic-pemphigus autoimmune response involves both intracellular and extracellular desmosomal antigens and suggest an overlapping distribution of autoantibody specificities among autoimmune bullous skin diseases. *Key words: paraneoplastic pemphigus/immunoelectron microscopy/immunoblot analysis. J Invest Dermatol 103:65–72, 1994*

Pemphigus refers to autoimmune blistering skin diseases caused by a variety of autoantibodies directed against intercellular junction systems [1]. Pemphigus vulgaris is characterized by the production of autoantibodies that recognize a 130-kD antigen, a member of the cadherin family [2], and pemphigus foliaceus, by autoantibodies directed against desmoglein [3,4]. Recently, a new type of pemphigus, associated with various neoplastic conditions, has been identified and termed paraneoplastic pemphigus [5].

Paraneoplastic pemphigus sera are characterized by their immunofluorescent staining pattern and the specificity of their autoimmune response: i) they label both the cell surface of keratinocytes and the basement-membrane zone of skin sections; ii) they bind to simple epithelial cells and non-epithelial tissues as well; iii) they immunoprecipitate an antigenic complex of 250-, 230-, 210-, and 190-kD polypeptides. The 230-kD band corresponds to the bullous pemphigoid antigen and the 250- and 210-kD proteins were recently identified as desmoplakins I and II [6]. The 190-kD band might correspond to an as yet unidentified epithelial antigen. So far, only a few patients with paraneoplastic pemphigus have been studied. In particular, the *in vivo* and *in vitro* ultrastructural localization of autoantibody deposits has not yet been reported.

The objective of this study was to use direct and indirect immunoelectron microscopy and immunoblotting to analyze the autoimmune response in five patients with clinical and histologic features and whose sera gave the immunofluorescence staining pattern typical of paraneoplastic pemphigus.

PATIENTS AND METHODS

Sera and Tissue Samples The five patients included in this study fulfilled the clinical and immunohistologic criteria proposed by Anhalt *et al* for the diagnosis of paraneoplastic pemphigus [5]. The patients had polymorphous skin eruptions with mucosal erosions. The common histologic picture consisted of keratinocyte necrosis and acantholysis. Direct immunofluorescence labeling of peribullous skin samples stained both the cell surface of the keratinocytes and the basement-membrane zone. The sera recognized stratified squamous epithelia, other epithelia, such as urinary bladder, small bowel and liver, and non-epithelial tissues (myocardium). When evaluated by indirect immunofluorescence on rat tongue sections, these sera contained anti-epithelial cell-surface antibodies at titers ranging from 1 : 20 to 1 : 1600. The associated neoplasms were the following: four non-Hodgkin's nodal lymphomas (patients 1, 2, 3, and 4) (three of them died within 1 to 5 months

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after diagnosis) and one adenocarcinoma of the colon (patient 5). Patient 4 was a 65-year-old female who presented with a erythema multiforme-like eruption with severe mucosal involvement. Her eruption was first diagnosed as possibly drug related, because of a histologic picture of keratinocyte necrosis. Diagnosis was subsequently re-evaluated because of the persistence of the eruption after treatment withdrawal and after the detection of an abdominal tumor corresponding to a diffuse mixed small- and large-cell non-Hodgkin's lymphoma. The eruption cleared rapidly under oral corticosteroid therapy (prednisone 1 mg/kg/d). The patient was also treated with a multiagent polychemotherapy regimen resulting in a partial remission of her lymphoma. Control sera were obtained from patients with pemphigus vulgaris 5, pemphigus foliaceus 3, bullous pemphigoid 3, and six healthy individuals.

Monoclonal Antibodies Two murine monoclonal antibodies (MoAb) specific for desmoplakins I–II and desmoglein I (Progen-Biotechnik, Heidelberg, Germany) and MoAb F12 were used as probes. F12 is a human IgM MoAb derived from a pemphigus vulgaris patient that recognizes a 185-kD polypeptide on immunoblotting using bovine tongue epithelium as the substrate [7]. MoAb F12 was demonstrated to bind to desmosomal plaques and hemidesmosomes by indirect immunoelectron microscopy [8]. Murine MoAb directed against human IgG subclasses were all purchased from Boehringer-Mannheim (SA, Meylan, France): anti-human IgG1 (clone NL16), anti-human IgG2 (clone GOM1), anti-human IgG3 (clone ZGA); and anti-human IgG4 MoAb (clone RJ4).

Indirect Immunofluorescence Analysis Rat tongue and normal human skin were frozen in liquid nitrogen and stored at −70°C until used. Four-micron-thick cryostat sections were cut and allowed to air-dry at room temperature. Unfixed sections were incubated with different dilutions of the paraneoplastic pemphigus sera in a moist chamber for 30 min and washed twice with phosphate-buffered saline (PBS). Then, skin samples were incubated with fluorescein/isothiocyanate-conjugated goat anti-human IgG1, IgG2, IgG3, or IgG4 diluted 1:20 in PBS for 40 min. After washing with PBS, the sections were examined under a Leitz Orthoplan fluorescence microscope equipped with standard filters.

Immunoelectron Microscopy Direct immunoelectron microscopy was performed on biopsies of peribullous skin from patients with paraneoplastic pemphigus 1, pemphigus vulgaris 3, pemphigus foliaceus 2, and bullous pemphigoid 3, as described previously [9]. Briefly, unfixed samples were sliced into 0.7-mm-thick sections, washed with agitation in Hanks' balanced salt solution (HBSS), and incubated for 22 h with peroxidase-labeled goat anti-human IgG or anti-C3 (Biosys, S.A., Compiègne, France) diluted 1:10 in HBSS. After washing, the skin samples were fixed in Karnovsky's fixative for 3 h at 4°C. The peroxidase activity was then revealed with dimethylarsinic acid buffer (Sigma Chemicals, Inc., St. Louis, MO) for 1 h at room temperature. After washing, tissue samples were post-fixed with 1% osmium tetroxide for 10 min, washed, dehydrated, and embedded in epoxy medium.

Indirect immunoelectron microscopy was performed with sera from patients with paraneoplastic pemphigus 5, pemphigus vulgaris 5, pemphigus foliaceus 3, bullous pemphigoid 3, from one normal human individual, and, with anti-desmoplakins I–II MoAb, using normal skin and/or oral mucosa as the substrates. Unfixed samples were prepared as described above and incubated with sera or anti-desmoplakins I–II MoAb diluted 1:10 in HBSS overnight at 4°C. After washing in HBSS, tissue samples were incubated with peroxidase-labeled goat anti-human whole IgG or goat anti-murine IgG (Nordic Immunology, Tilburg, The Netherlands) for 22 h at 4°C. For indirect immunoelectron microscopy analysis of autoantibody IgG subclasses, tissue samples were first incubated with patients' sera, washed in HBSS, and then incubated with murine anti-human IgG1, IgG2, IgG3, or IgG4 MoAb. After washing, samples were incubated with peroxidase-labeled goat anti-murine Ig. Immunoperoxidase staining and visualization were then performed as described above. When performed counterstaining is indicated in the figure legends.

Immunoblot Analysis

Bovine Tongue Extraction: Bovine tongue was obtained from a freshly slaughtered animal. The epithelium with its attached connective tissue was minced finely with scissors and extracted with ice-cold 0.0625 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS) in the presence of phenylmethylsulfanyl fluoride (Sigma) and 10 ng/ml of each of the following protease inhibitors: leupeptin, antipain, chymostatin, and pepstatin (Sigma). The suspension was ultrasonicated at setting 6 for 15 seconds (microultrasonic cell disrupter) and centrifuged at 1500 rpm for 1 h at 4°C. The resulting supernatant was boiled for 2 min and aliquots were frozen at −70°C.

Human Epidermal Extraction: Dermolipectomy specimens of normal human skin were used as the source of epidermis. To separate the dermis from epidermis, 4–25 cm^2 skin samples were incubated in saline at 56°C for 30 seconds, after which the epidermis was removed as previously described [10]. Approximately 25 cm^2 of epidermis was placed in 1 ml of sample buffer: 65 mM Tris-HCl, 10 mM ethylenediamine tetraacetic acid, 2 mM phenylmethylsulfanyl fluoride, 2% SDS, pH 6.8, containing 0.01 mg/ml of each of the protease inhibitors pepstatin, antipain, leupeptin, and chymostatin. The homogenate was vortexed (1 min), ultrasonicated at 4°C (4 × 15 seconds) and then centrifuged at 1500 rpm for 20 min. The supernatant was frozen at −30°C until it was used for electrophoresis.

Immunoblotting Procedure: A 6% separative gel was used for SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were electrophoretically transferred onto nitrocellulose filters (24 V for 2 h) in 12.5 mM Tris-OH, 96 mM glycine buffer with 20% (v/v) methanol. Then, the remaining binding sites on the filter replica of the gel were saturated by immersion for 1 h at room temperature in PBS containing 5% (v/v) milk powder. Longitudinal strips of the filter were cut and incubated for 1 h with either a 1:40 dilution of the test serum or with a 1:100 dilution of anti-desmoglein I, anti-desmoplakin I–II MoAb, or MoAb F12 in PBS containing 5% milk powder and 0.1% Tween 20. After three washes in PBS, the strips were subsequently incubated with biotinylated goat anti-human or anti-mouse Ig (Caltag Laboratories, San Francisco, CA) diluted 1:1000 in PBS-0.1% Tween 20, 5% milk powder for 1 h at room temperature. After washing with PBS-0.1% Tween 20, the strips were incubated with alkaline phosphatase-conjugated streptavidin (Caltag Laboratories), washed with PBS, and revealed with D-aminobenzidine (Sigma).

For Ig subclass studies, the replicas were incubated with the patients' or control sera; washed; exposed to the anti-human IgG1, IgG2, IgG3, or IgG4 murine MoAb; and, finally, incubated with biotinylated-goat anti-mouse Ig.

Affinity Purification of Paraneoplastic Pemphigus IgG: Affinity purification of anti-130-kD paraneoplastic pemphigus IgG was performed according to a previously described method [11]. Briefly, horizontal strips of nitrocellulose containing the 130-kD band were cut out, incubated with the paraneoplastic pemphigus serum from patient 4 or a pemphigus vulgaris serum, and washed. Bound antibodies were eluted with sodium citrate buffer, pH 3.2, immediately neutralized, and used for indirect immunofluorescence experiments as described above.

RESULTS

Immunologic Procedures Using Anti-Human Whole Ig as the Tracer

Immunoblot Analysis: Sera from the five paraneoplastic pemphigus patients were analyzed by western blotting using bovine tongue epithelium as the substrate and anti-human whole IgG as the tracer (Fig 1*a,b*). All these sera recognized the 250- and 220-kD bands that comigrated with those identified by the murine MoAb to desmoplakins I–II. Four of the five sera recognized a 185-kD band (or a 180–190-kD doublet, depending upon the immunoblotting run) comigrating with the polypeptide identified by MoAb F12. None of the five paraneoplastic pemphigus sera or the bullous pemphigoid sera bound to the 230-kD polypeptide of the bullous pemphigoid antigen.

Because bovine tongue extract was shown to be an inappropriate substrate for the detection of bullous pemphigoid antigen, western blotting was performed using human epidermal extracts as the antigen. Two paraneoplastic pemphigus sera were tested and both recognized a 230-kD band that comigrated with the band recognized by bullous pemphigoid sera (data not shown). Thus, immunoblotting analysis of paraneoplastic pemphigus sera using anti-human whole Ig showed a pattern close to that obtained using an immunoprecipitation procedure [5].

Immunoelectron Microscopy: DIRECT IMMUNOELECTRON MICROSCOPY Direct immunoelectron microscopy study was performed on peribullous skin samples obtained from one patient with paraneoplastic pemphigus. Peroxidase labeling was localized exclusively in the basal and spine-cell layers of the epidermis. The peroxidase deposits were thick and localized to the part of the keratinocyte membrane associated with desmosomes (Fig 2*A*). Some keratinocytes exhibited features of cell degeneration and cell necrosis. Peroxidase de-

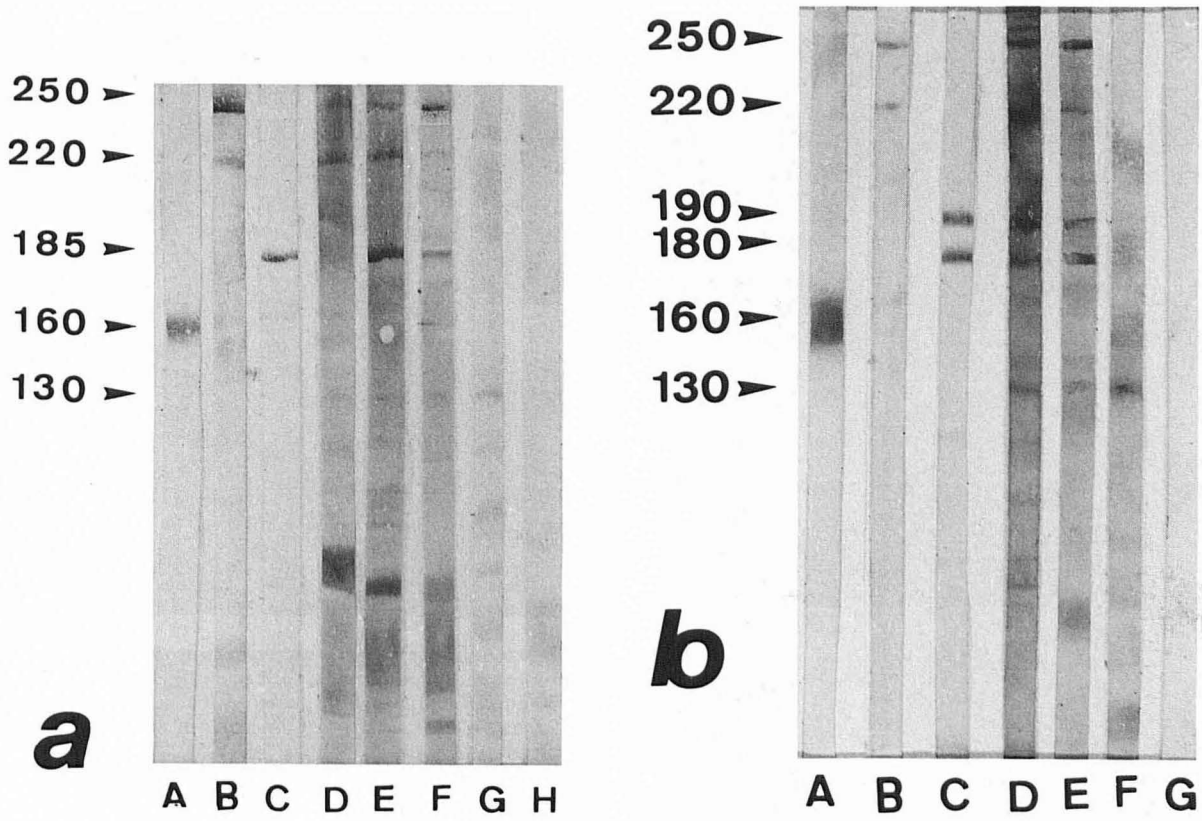


Figure 1. Immunoblot analysis of sera from paraneoplastic pemphigus patients. Bovine tongue epithelium extracts were used as the antigen and anti-human whole Ig as the tracers. Two different runs, *a* and *b*, are shown. *a) Lane A,* anti-desmoglein MoAb; *lane B,* MoAb to desmoplakins I–II; *lane C,* MoAb F12; *lanes D, E, F,* sera from paraneoplastic pemphigus patients 1, 2, and 3; *lane G,* serum from pemphigus vulgaris patient; *lane H,* serum from a healthy subject. *b) Lane A,* anti-desmoglein MoAb; *lane B,* MoAb to desmoplakins; *lane C,* MoAb F12; *lanes D* and *E,* sera from paraneoplastic pemphigus patients 4 and 5; *lane F,* serum from a pemphigus vulgaris patient; *lane G,* serum from a healthy subject.

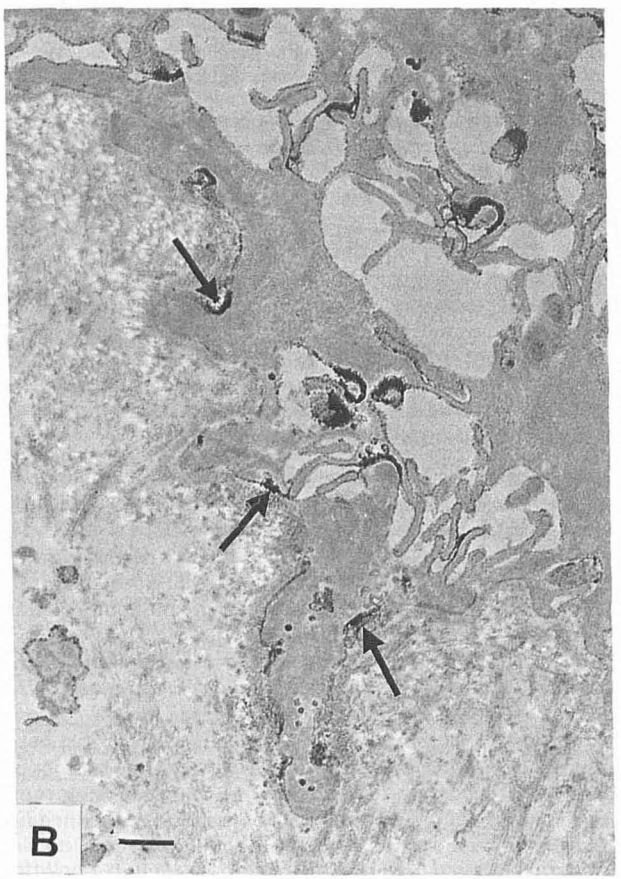
posits were seen on desmosomes and hemidesmosomes at the dermal-epidermal junction (Fig 2*B*); this pattern was not observed on the skin samples obtained from the three pemphigus vulgaris or the two pemphigus foliaceus patients studied (Fig 2*C*).

INDIRECT IMMUNOELECTRON MICROSCOPY Sera from the five patients with paraneoplastic pemphigus were characterized by indirect immunoelectron microscopy on normal human skin and/or oral mucosa specimens. They all strongly labeled the dermal-epidermal junction. The positive reaction was localized on hemidesmosomes, whereas no peroxidase deposits were seen in the adjacent keratinocyte basal membrane or the lamina lucida (Fig 3*A*). Figure 3*B* shows the labeling pattern of the keratinocyte plasma membrane of basal- and spine-cell layers. Peroxidase deposits predominated on desmosomes but were also present along the plasma cell membrane between desmosomes. Examination at higher magnification clearly demonstrated peroxidase deposits on the intracellular desmosomal plaques and, surprisingly, the extracellular part of desmosomes (Fig 3*C*). This staining pattern was constantly observed with the sera from the five paraneoplastic pemphigus patients. Pemphigus vulgaris sera labeled the extracellular part of the desmosome and the keratinocyte plasma membrane between desmosomes but did not react with hemidesmosomes and desmosomal plaques (Fig 3*D*).

Immunologic Procedures Using MoAb to Human IgG Subclasses as the Tracers Although immunoblotting results were consistent with those reported by others [5], immunoelectron microscopy labeling studies demonstrated that autoantibodies present in paraneoplastic pemphigus sera were directed not only against the intracellular components of desmosomes, but also against the desmoglea; this latter localization does not correspond to an autoantibody specificity previously identified in paraneoplastic pemphigus sera. Moreover, when we retrospectively reexamined our immunoblotting replica, we noticed that a barely visible 130-kD band (Fig 1*b, lanes D* and *E*) was recognized by two paraneoplastic pemphigus sera; this band comigrated with the band recognized by pemphigus vulgaris serum (Fig 1*b, lane F*). These results prompted us to undertake a second series of experiments, in which immunoblotting and immunoelectron microscopy analyses were performed using MoAb to human IgG subclasses as the probes, a procedure that enabled us to analyze the autoimmune response of these two sera more precisely. The results described below concern only patient 4. Table I summarizes data obtained in patients 4 and 5. Unfortunately, no more serum was available to undertake a similar study in the other three paraneoplastic pemphigus patients.

Immunoblot Analysis: By immunoblotting, IgG1 autoantibodies bound to the 250- and 220-kD bands that comigrated with those recognized by the murine MoAb to desmoplakins I–II, and two 140- and 110-kD bands that might correspond to degradation products of desmoplakins. Anti-IgG2 antibodies did not recognize any autoantibody labeling of the extract. IgG3 autoantibodies identified a 180–190-kD doublet that comigrated with the bands recognized by MoAb F12. Interestingly, the serum of paraneoplastic-pemphigus patient 4 contained IgG4 autoantibodies that bound to a 130-kD band that comigrated with the band recognized by IgG4 autoantibodies present in pemphigus vulgaris sera (Fig 4). Three bullous pemphigoid sera, three pemphigus foliaceus sera, and six sera from healthy individuals were tested at a 1:40 dilution, using murine MoAb to human IgG4 subclass. None of these sera contained IgG4 antibodies that bound to a 130-kD band.

Indirect Immunofluorescence Studies: By indirect immunofluorescence on rat tongue sections, paraneoplastic pemphigus IgG1 auto-



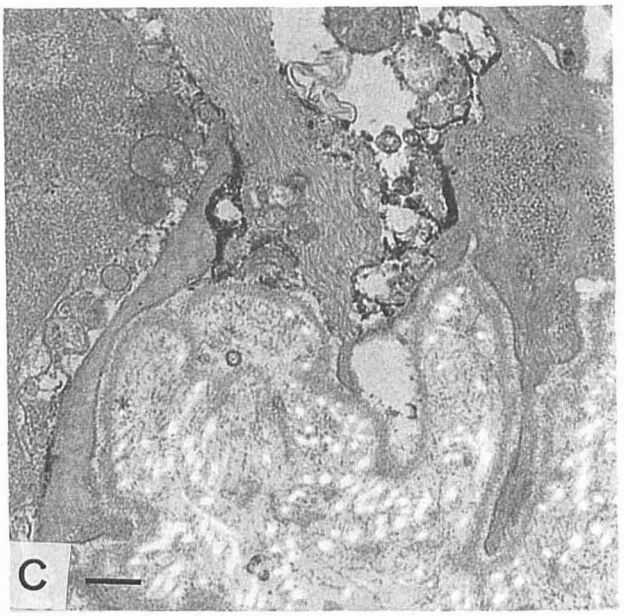


Figure 2. Direct immunoelectron microscopy on peribullous skin samples from paraneoplastic pemphigus patient 4. *A)* Labeled desmosomes (*bar,* 150 nm). *B)* Peroxidase deposits on the hemidesmosomes (*arrows*) (*bar,* 420 nm). *C)* Dermal-epidermal junction of skin samples from a pemphigus vulgaris patient (*bar,* 350 nm).

antibodies gave a pericytoplasmic fluorescence of keratinocytes with a pattern similar to that obtained with anti-desmoplakin MoAb. As for the immunoblots, IgG2 antibodies did not recognize an autoantibody labeling of the section. IgG3 paraneoplastic pemphigus antibodies gave a patchy fluorescence of the keratinocyte cell membrane and of the basement-membrane zone, the same topographic distribution as that obtained with MoAb F12. Paraneoplastic pemphigus IgG4 autoantibodies gave a thin line of label, exclusively in the cell surface of the keratinocytes with no straining of the bazement membrane zone, as did the IgG4 autoantibodies present in a pemphigus vulgaris serum (data not shown).

Indirect Immunoelectron Microscopy: Indirect immunoelectron microscopy experiments performed on normal human oral mucosa using murine MoAb to human IgG subclasses confirmed the data obtained by indirect immunofluorescence and western blot analyses. Paraneoplastic pemphigus IgG1 autoantibodies bound to the desmosomal plaques of keratinocytes and did not recognize the extracellular part of the desmosomes (Fig 5*A*). The pattern given by paraneoplastic pemphigus IgG3 autoantibodies (Fig 5*B*) was similar to that obtained with MoAb F12, which labels the desmosomal plaques and hemidesmosomes [7]. Paraneoplastic pemphigus IgG4 autoantibodies identified the desmoglea with a slight spread of the peroxidase deposits along the keratinocyte membrane between desmosomes (Fig 5*C*) but never bound to hemidesmosomes or the desmosomal plaques, a pattern similar to that given by IgG4 pemphigus vulgaris autoantibodies.

Affinity Purification of Paraneoplastic Pemphigus Anti–130-kD IgG4 Antibodies: To confirm the specificity of paraneoplastic pemphigus IgG4 antibodies that bound to the 130-kD band, affinity-purified IgG were obtained from paraneoplastic pemphigus patient 4, one pemphigus vulgaris patient, and one normal control sera, by elution of antibodies bound to the 130-kD band. Affinity-purified IgG were then tested by indirect immunofluorescence on rat tongue sections, using anti-human IgG4 murine MoAb. Affinity-purified anti–130-kD IgG4 antibodies from paraneoplastic pemphigus patient 4 and from the pemphigus vulgaris patient stained the cell

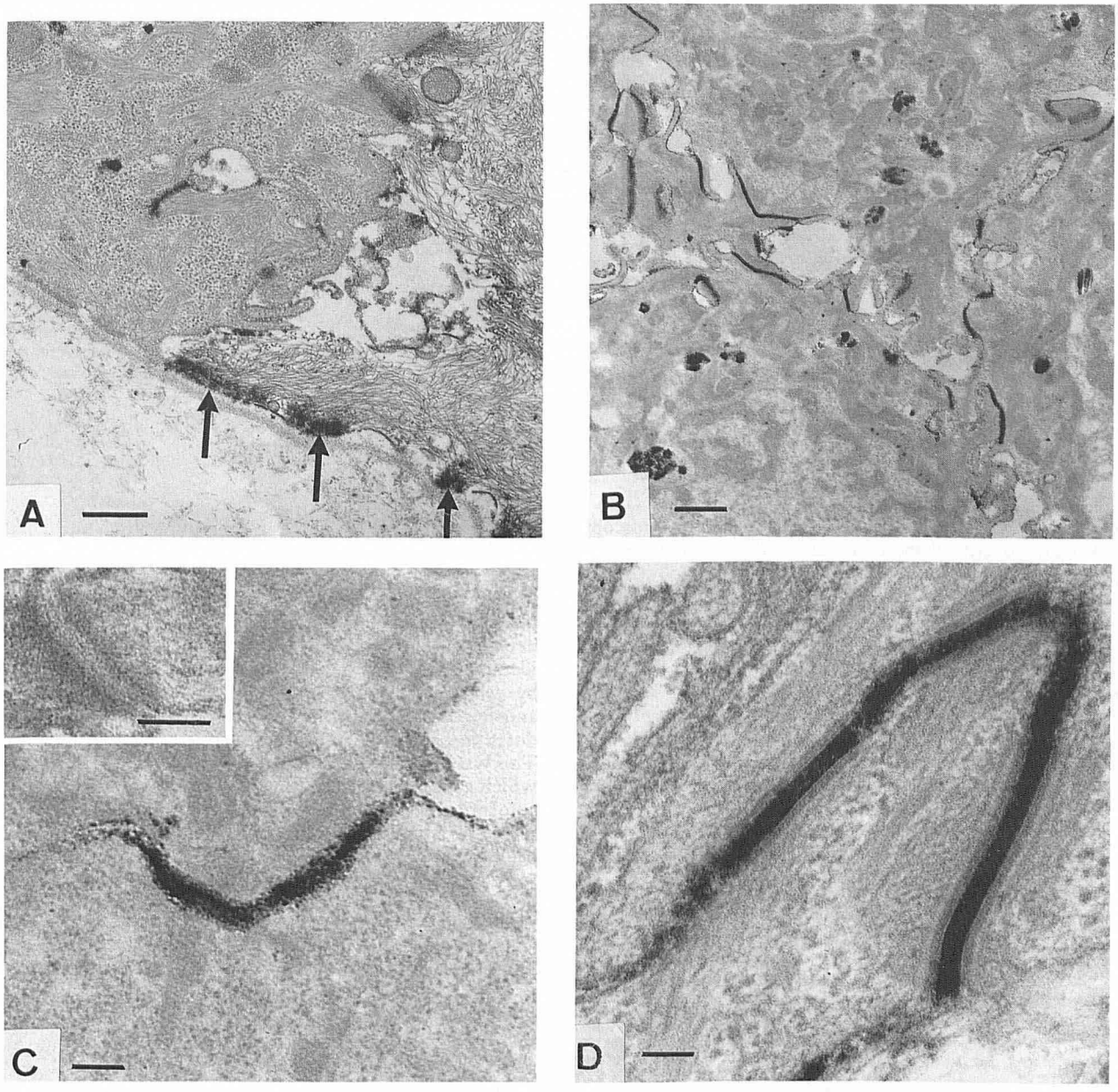


Figure 3. Indirect immunoelectron microscopy on normal oral mucosa using serum from paraneoplastic pemphigus patient 2. *A)* Examination of the dermal-epidermal junction on this counterstained grid showed peroxidase deposits on hemidesmosomes (*arrows*) (*bar,* 220 nm). *B)* Labeling of the desmosomal regions of the keratinocyte plasma membrane (*arrows*) (*bar,* 350 nm). *C)* Desmosome at higher magnification (*bar,* 45 nm); *inset:* negative control experiment using serum from a healthy subject (*bar,* 60 nm). *D)* Indirect immunoelectron microscopic pattern observed with a pemphigus vulgaris patient's serum on normal oral mucosa (*bar,* 20 nm).

surface of the keratinocytes, whereas no staining of the bazement membrane zone was observed (Fig 6).

DISCUSSION

The major observation of this study is the demonstration that the autoimmune response in paraneoplastic pemphigus is directed not only against antigenic determinants of hemidesmosomes and the cytoplasmic plaques of desmosomes but also against components of the extracellular part of desmosomes.

Paraneoplastic pemphigus is a recently identified entity characterized by clinical and histologic features and the specificity of the autoimmune response [5]. The target antigens have been identified by immunoprecipitation [5] and by immunoblotting [6] and include desmoplakins I and II, the 230-kD antigen of bullous pemphigoid, and an as yet unidentified antigen of 190 kD. In addition, we have previously shown that paraneoplastic pemphigus sera also contain an autoantibody population binding to a 185-kD polypeptide recognized by a human MoAb (MoAb F12) derived from the peripheral blood lymphocytes of a 14-year-old patient with pemphigus vulgaris [7,8]. MoAb F12 labeled the desmosomal plaques of keratinocytes by indirect immunoelectron microscopy [8]. Thus, in light of these results, paraneoplastic pemphigus might be considered a unique disorder characterized by autoimmune response mainly directed against the intracellular components of junction systems. This concept was supported by our immunoblotting study using anti-human whole Ig to visualize the polypeptides recognized by our five paraneoplastic pemphigus sera because they bound to the 250- and 220-kD bands of desmoplakins I–II and to the 185-kD band defined by MoAb F12, when bovine tongue extracts were used as the antigen, and to the 230-kD band of bullous pemphigoid, when immunoblotting was performed on human epidermal extracts.

Table I. Immunoblotting, Indirect Immunofluorescence, and Indirect Immunoelectron Microscope Analyses of Sera from Paraneoplastic Pemphigus Patients 4 and 5, Using Anti-IgG Subclass MoAb as Tracers

MoAb to Subclass	Immunoblotting (kD band)[a]		Indirect Immunofluorescence[b]		Indirect Immunoelectron Microscopy[c]	
	4	5	4	5	4	5
IgG1	250–220	250–220 180–190 130	Keratinocyte membrane	Keratinocyte membrane Bazement membrane zone	Desmosomal plaques	Desmosomal plaques Desmoglea Hemidesmosomes
IgG2	—	—	—	—	—	—
IgG3	180–190	—	Keratinocyte membrane Basement membrane zone	—	Desmosomal plaques Hemidesmosomes	—
IgG4	130	250–220 180–190 130	Intercellular substance	Keratinocyte membrane Basement membrane zone	Desmoglea	Desmosomal plaques Desmoglea Hemidesmosomes

[a] Bovine tongue extracts used as the antigen.
[b] Rat tongue sections used as the substrate.
[c] Normal human oral mucosa used as the substrate.

However, direct and indirect immunoelectron microscopy images were not consistent with this view. Indeed, paraneoplastic pemphigus autoantibodies bound to hemidesmosomes (which may correspond to the anti-230-kD or the anti-185-kD antibodies) [8,12] and to desmosomal plaques (which may correspond to antidesmoplakins I and II and anti-185-kD antibodies). In addition, all sera tested always bound to the extracellular part of desmosomes and to the keratinocyte plasma membrane, which does not contain the antigenic structures targeted by the autoantibody populations so far identified in paraneoplastic pemphigus sera. In this regard, it is interesting to note that skin samples obtained from neonatal BALB/c mice injected with sera from paraneoplastic pemphigus patients exhibited a diffuse IgG binding along the keratinocyte membrane.†

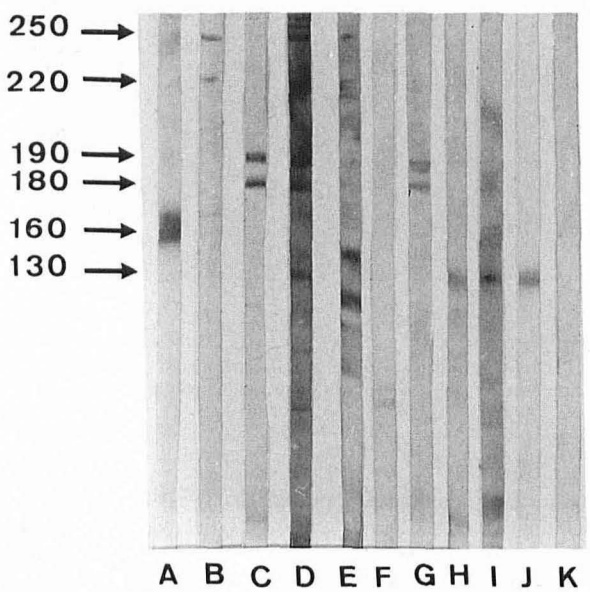
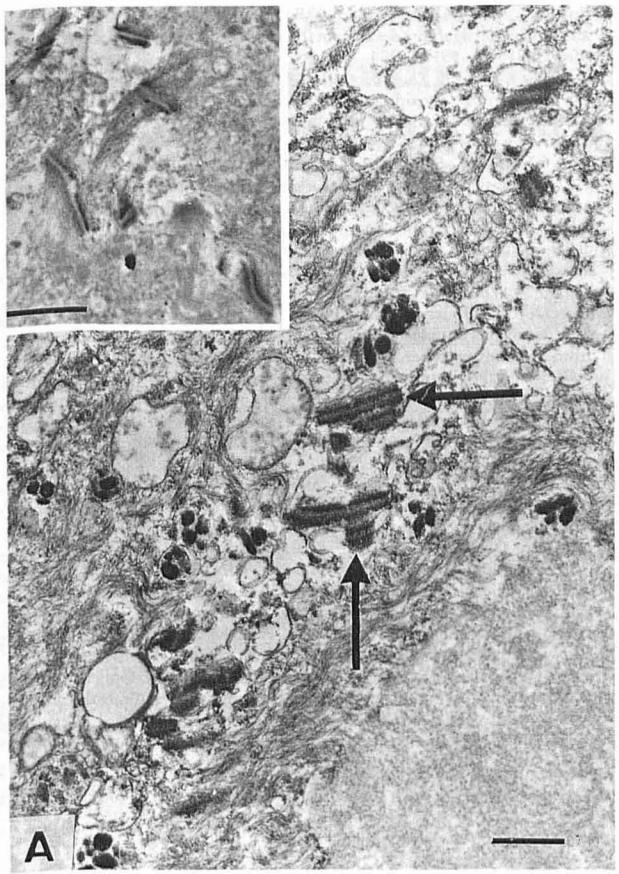


Figure 4. Immunoblot analysis of serum from paraneoplastic pemphigus patient 4 using bovine tongue extracts as the antigen and MoAb to human IgG subclasses as the tracers. *Lane A,* anti-desmoglein MoAb; *lane B,* MoAb to desmoplakins; *lane C,* MoAb F12; *lane D,* serum from paraneoplastic pemphigus patient 4 analyzed with anti-human whole IgG; *lane E,* IgG1 antibodies from paraneoplastic pemphigus patient 4; *lane F,* IgG2 antibodies from this serum; *lane G,* IgG3 antibodies from the paraneoplastic pemphigus serum 4; *lane H,* paraneoplastic pemphigus IgG4 antibodies; *lane I,* serum from a pemphigus vulgaris patient analyzed with anti-human whole Ig; *lane J,* identification of IgG4 autoantibodies in this pemphigus vulgaris serum; *lane K,* serum from a healthy subject.

This observation raises the question of the presence of an as of yet unrecognized autoantibody population in paraneoplastic pemphigus sera. Immunoblotting experiments using anti-human whole Ig demonstrated that two paraneoplastic pemphigus sera very weakly identified a 130-kD band that comigrated with the band recognized by pemphigus vulgaris sera. Further analysis of these sera using anti-human IgG subclasses in the different immunologic procedures enabled us to better characterize the autoimmune response. This was particularly demonstrative with the serum of patient 4, in which each IgG subclass had a unique antigenic specificity: IgG1 autoantibodies gave a pericytoplasmic immunofluorescence staining pattern on rat tongue section, bound to the 250- and 220-kD bands by immunoblotting, and recognized exclusively the desmosomal plaques by immunoelectron microscopy. The IgG3 autoantibodies of patient 4 had much in common with MoAb F12: they had the same topographical distribution, i.e., they stained the keratinocyte membrane and basement-membrane zone; they bound to the 180–190-kD doublet; they stained the desmosomal plaques and the hemidesmosomes. The most important finding emerged from the study of IgG4 autoantibodies: on immunoblots, they bound to the 130-kD polypeptide that comigrated with IgG4 autoantibodies present in pemphigus vulgaris sera and recognized an antigen located in the desmoglea.

Thus, indirect immunoelectron microscopy and immunoblotting strongly suggested that paraneoplastic pemphigus sera contain antibodies directed against extracellular desmosomal components and some against a 130-kD band that might correspond to the pemphigus vulgaris antigen. This latter presumption is supported by the results of the indirect immunofluorescence experiments using affinity-purified anti-130-kD IgG4 antibodies, which showed that this material stained the cell surface of keratinocytes on rat tongue section in a manner similar to that given by anti-130-kD IgG4 antibodies purified from a pemphigus vulgaris serum. This raises the question why such autoantibody populations have not been recognized in previous studies of paraneoplastic pemphigus sera [5,6]. First, technical procedures may account for this discrepancy. Our immunoelectron microscopy experiments, which used unfixed tissue samples (oral mucosa), may favor the detection of autoantibody populations directed against extracellular desmosomal components because, on this substrate, desmosomal plaques appeared much less accessible than desmoglea. Second, anti-130-kD antibodies in

† Futamura S, *et al:* Ultrastructural studies of acanthosis induced in vivo by passive transfer of paraneoplastic pemphigus IgG (abstr). *J Invest Dermatol* 98:586, 1992.



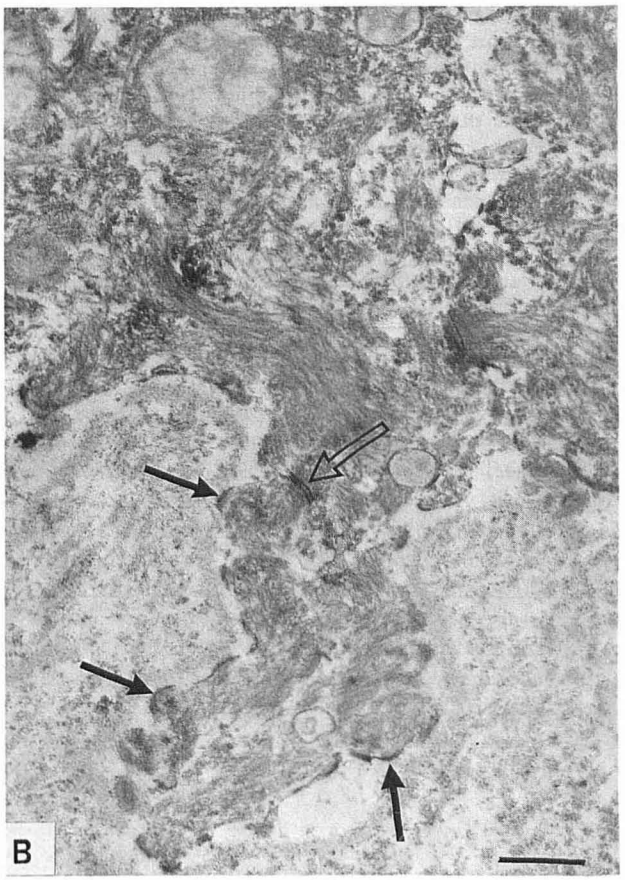
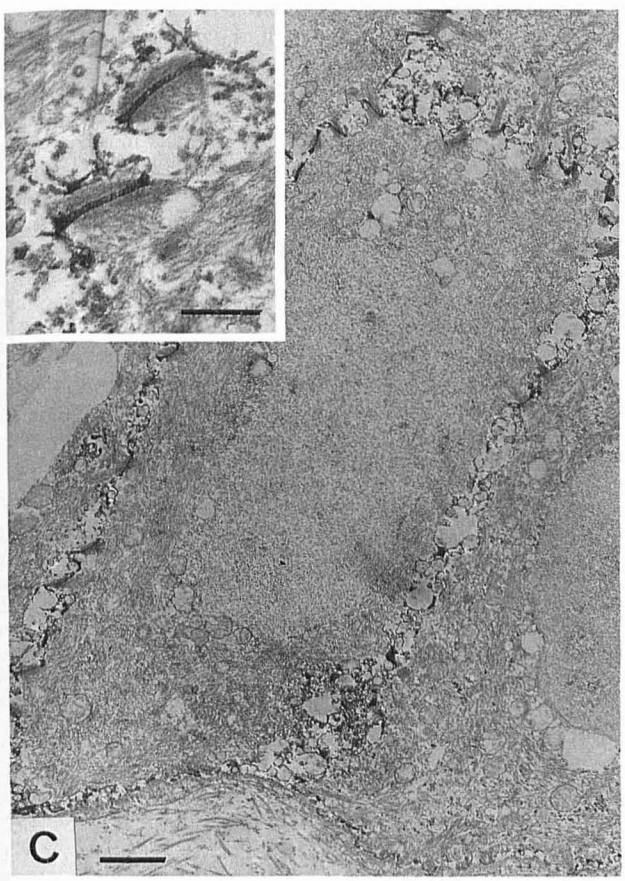


Figure 5. Indirect immunoelectron microscopic study of labeling of normal human oral mucosa by a paraneoplastic pemphigus (patient 4) serum recognized by MoAb to human IgG subclasses and visualized by an immunoperoxidase reaction. *A)* IgG1 autoantibodies labeled exclusively the desmosomal plaques (*arrows*) (*bar,* 400 nm); *inset:* anti-desmoplakin MoAb (*bar,* 400 nm). *B)* IgG3 autoantibodies from the same paraneoplastic pemphigus serum labeled desmosomal plaques (*open arrow*) and hemidesmosomes (*black arrow*) (*bar,* 960 nm). *C)* The fine line of peroxidase deposits obtained with paraneoplastic pemphigus IgG4 autoantibodies (*bar,* 1.5 μm); *inset,* desmosomes at higher magnification (*bar,* 400 nm).



paraneoplastic pemphigus sera may constitute a minor component of the autoimmune response. Results obtained with our immunoblotting procedure support this hypothesis. With alkaline phosphatase–labeled anti-human whole Ig, the 130-kD band was barely visible with two paraneoplastic pemphigus sera, whereas a two-step procedure that applied, sequentially, a murine anti-human IgG subclass MoAb and alkaline phosphatase–labeled anti-mouse Ig and thus amplified the reaction clearly, revealed the 130-kD band. Third, since three sera stained the intercellular part of the desmo-

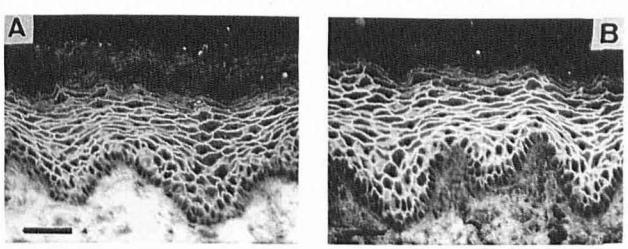


Figure 6. Indirect immunofluorescence study on rat tongue sections, of affinity-purified IgG antibodies from *(A)* paraneoplastic pemphigus patient 4's serum (*bar,* 60 μm); *(B)* pemphigus vulgaris serum (*bar,* 60 μm). Murine anti-human IgG4 antibody was used as the tracer.

some but failed to react with the 130-kD band, one may hypothesize that in some paraneoplastic pemphigus sera, the keratinocyte cell surface staining is produced by an autoantibody population different from the anti-130-kD antibodies. In this regard, a recent study suggests that paraneoplastic pemphigus sera recognized a 170-kD antigen corresponding to a transmembrane glycoprotein.‡

Taken together, these results clearly emphasize the heterogeneity of the autoimmune response observed in patients with paraneoplastic pemphigus, which targets both the desmosomal plaques, the desmoglea, and hemidesmosomes, and suggest an overlapping distribution of autoantibody specificities among autoimmune bullous skin diseases.

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‡ Anhalt GJ, Hilu J, Takehala K, Lee A, Casciola-Rosen L: Identification of a putative transmembrane autoantigen in paraneoplastic pemphigus (abstr). *J Invest Dermatol* 100:508, 1993.